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JC Rec'd PCT/PTO 08 NOV 2001

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COMPOSITIONS AND METHODS FOR IMPROVING LEARNING AND MEMORY

This application claims priority to U.S. Provisional Application No. 60/133,371, filed May 13, 1999, the entirety of which is incorporated by reference herein.

FIELD OF THE INVENTION

This invention relates to the field of neurobiology. In particular, the invention provides a method to improve learning and memory in humans and animals, through the modification of the NMDA receptor.

BACKGROUND OF THE INVENTION

Several publications are referenced in this application to more fully describe the state of the art to which this invention pertains. The disclosure of each such publication is incorporated by reference herein.

It has been hypothesized that memory storage in the mammalian brain involves modifications of the synaptic connections between neurons. Hebb's rule (1949) of "correlated activity" holds that both learning and memory are based on modifications of synaptic strength among neurons that are co-active, i.e., when presynaptic and postsynaptic neurons are active simultaneously, their connection becomes strengthened.

It has been postulated that *N*-methyl-D-aspartate (NMDA) receptors can implement the Hebb rule at the synaptic level. For this reason, they are considered good candidates as synaptic elements for the induction of activity-dependent synaptic plasticity. NMDA receptors

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act as coincidence detectors because they require both presynaptic activity (glutamate released by axonal terminals) and postsynaptic activity (depolarization that releases the Mg^{2+} block) as condition for channel opening. Active NMDA receptor channels allow calcium influx into the postsynaptic cell, which triggers a cascade of biochemical events resulting in synaptic change.

The NMDA receptors are heteromeric complexes consisting of NR1 and various NR2 subunits (Nakanishi, *Science* **258**, 597-603, 1992; Hollmann & Heinemann, *Annu. Rev. Neurosci* **17**, 31-108, 1994). The NR1 subunit is essential for channel function, whereas the NR2 subunit regulates channel gating and Mg^{2+} dependency (Monyer et al., *Science* **256**, 1217-21, 1992). In the adult forebrain regions such as the hippocampus and the cortex, only NR2A and NR2B subunits are available to form the receptor complex with NR1 subunit. The recombinant NR1-NR2B complex *in vitro* has a longer duration of excitatory postsynaptic potentials than those of the NR1-NR2A complex (Monyer et al., *Neuron* **12**, 529-40, 1994). NR2B expression is down-regulated during the transition period from juvenile to adulthood (Sheng et al., *Nature* **368**, 144-147, 1994; Okabe et al., *J. Neurosci.* **18**, 4177-88, 1998), correlating with the gradual shortening of the NMDA channel duration (Carmignoto & Vicini, *Science* **258**, 1007-11, 1992; Hestrin, *Neuron* **9**, 991-9, 1992).

The phenomena of long term potentiation (LTP) and long term depression (LTD) have been a focus of studies on neural plasticity at the molecular level. These terms refer to multiple mechanisms involved in altering the strength of synapses. The induction of LTP requires, at least in one form, the activation of NMDA receptors. It is believed that NMDA receptor-dependent LTP is elicited by giving a strong pattern of electrical

stimulation (i.e., a 25-100 Hz train for ~1 sec) to the inputs, which triggers a rapid and lasting increase in synaptic strength.

The hippocampus is the most intensely studied region for the importance of NMDA receptors in synaptic plasticity and memory. It is known that lesions of the hippocampus in humans and other mammals produce severe amnesia for certain memories (see review of Squire, 1987). Disruption of NMDA receptors in the hippocampus has been shown to lead to blockade of synaptic plasticity and also to memory malfunction (reviewed by Morris et al., 1991; Rawlins, 1996). For instance, application of NMDA receptor antagonists completely blocks the induction of LTP in most hippocampal synapses. Rats that received infusion of such antagonists into the brain were deficient in performing certain spatial memory tasks (Morris et al., 1986). Similar results were observed in genetically engineered knockout mice which lacked a gene encoding a component believed to be downstream of activated NMDA receptors in the biochemical cascade for LTP induction (Silva et al., 1992a; Silva et al., 1992b; reviewed by Chen & Tonagawa, 1997).

Although the foregoing results circumstantially implicate NMDA receptor-induced LTP in the hippocampus as critical for certain types of memory and learning, they are equivocal, due to the non-specific nature of the respective treatments. For instance, in the case of the gene knockout mice, every cell in the animals lacks the gene of interest, thereby affecting all functions of the gene product, not just LTP induction. Likewise, in pharmacological studies, the target of NMDA receptor antagonist infusion was not restricted to the hippocampus; therefore, NMDA receptors expressed in neurons of the neighboring neocortex and other brain areas were also inhibited to a varying extent. Moreover,

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this non-restricted binding has often been observed to produce sensory and motor disturbances.

To circumvent some of the interpretational difficulties arising from the lack of specificity in the aforementioned gene knockout or pharmacological results, a NMDA receptor knockout mouse was developed that comprised deletion of the NR1 subunit specifically in the pyramidal cells of the hippocampal CA1 region (Tsien et al., *Cell* **87**, 1317-26, 1996). These knockout mice were demonstrated to lack NMDA receptor-mediated synaptic currents and LTP in the CA1 region and exhibited impaired spatial memory, but unimpaired nonspatial learning (Tsien et al., *Cell* **87**, 1327-38, 1996). Multiple electrode recording techniques revealed that, although the CA1 pyramidal cells of the mice retained place-related activity, there was a significant decrease in the spatial specificity of individual place fields. Moreover, there was a striking deficit in the coordinated firing of pairs of neurons tuned to similar spatial locations (McHugh et al., *Cell* **87**, 1339-49, 1996). These results demonstrated that NMDA receptor-mediated synaptic plasticity is necessary for the proper representation of space in the CA1 region of the hippocampus, and strongly suggested that activity-dependent inhibition of CA1 synapses, as mediated by NMDA receptors, plays an essential role in the acquisition of spatial memories.

The foregoing results obtained with NMDA receptor knockout mice provide evidence that complete inhibition of the receptor in a specific region of the brain inhibits certain specific forms of memory. However, they do not address the role of the receptor in broader types of learning and memory, nor do they provide direction in applying such information to the improvement of learning and memory, or to treatment of diseases or disorders that detrimentally affect learning and memory.

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Such neuronal degenerative disorders, which include for example, Alzheimer's disease and stroke, are becoming increasingly prevalent in the population as it ages. It would be a significant advance in the field of clinical neurobiology to devise a means by which such disorders could be treated or reversed, and which further could be applied to the improvement of learning and memory in any individual in need of such treatment.

SUMMARY OF THE INVENTION

The present invention provides methods and tools for improving learning or memory in a subject, for treating learning and memory-related degenerative disease in a patient in need of such treatment, for identifying novel agents capable of regulating learning and memory (positively or negatively), and for identifying genes involved in biological processes related to learning and memory.

In accordance with one aspect of the invention method for improving learning or memory in a subject is provided. The method comprises modifying NMDA receptors in neural synapses of the subject's brain, such that the NMDA receptor function is increased as compared with an equivalent unmodified subject. Such an increase in NMDA receptor function is measured as an increase in synaptic plasticity and NMDA receptor activation (i.e., increase in channel decay time or peak amplitude).

In accordance with another aspect of the invention, a method of treating a neurodegenerative disorder affecting learning or memory in a patient in need of such treatment is provided. The method comprises modifying NMDA receptors in neural synapses of the patient's brain, such that the NMDA receptor function is increased as compared with an equivalent unmodified patient, the modification resulting in improved learning

or memory in the patient.

According to another aspect of the invention, a genetically altered non-human animal having enhanced learning and memory as compared with an equivalent, but unaltered animal, is provided. The genetic alteration results in a modification of NMDA receptors in neural synapses of the animal's brain, such that the NMDA receptor function is increased as compared with an equivalent unaltered animal.

According to another aspect, the invention provides a transgenic non-human animal that expresses an NR2B transgene in its brain. In a preferred embodiment, the animal is a rodent, most preferably a mouse.

According to another aspect of the invention, a method of identifying compounds that enhance learning and memory in a subject by increasing expression of NR2B genes in the subject is provided. The method comprises providing a chimeric DNA construct comprising an NR2B promoter operably linked to a reporter gene, contacting the chimeric DNA construct with a test compound suspected of up-regulating the NR2B promoter, and measuring expression of the reporter gene, an increase in the expression being indicative that the test compound enhances learning and memory in the subject by increasing expression of NR2B genes in the subject.

According to yet another aspect of the invention, an *in vitro* or *in vivo* assay for identifying compounds that enhance learning and memory in a subject by affecting NMDA receptor function is provided. The *in vitro* method comprises: (a) providing a pair of cell cultures, one being transgenic and expressing an exogenous nucleic acid molecule encoding NR2B, and the other being non-transgenic for expression of an exogenous nucleic acid molecule encoding NR2B; (b) treating the non-transgenic cells with a test compound suspected to

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affect the expression NR2B or activity of the NMDA receptors; and (c) comparing NMDA receptor function of the treated, non-transgenic cells with NMDA receptor function of the transgenic cells and, optionally, NMDA receptor function of an untreated, non-transgenic cells, a change in NMDA receptor function in the treated, non-transgenic cells that comprises the same features of NMDA receptor function exhibited in the transgenic cells being indicative that the test compound enhances learning and memory in a subject by affecting NMDA receptor function.

The *in vivo* method is practiced similarly, but instead of cultured cells, transgenic and non-transgenic animals are used.

According to still another aspect of the invention, another *in vitro* or *in vivo* assay for identifying compounds that enhance function of NMDA receptors in a subject is provided. The *in vitro* assay comprises: (a) providing a pair of cell cultures; (b) treating one of the cell cultures with a test compound suspected of enhancing NMDA receptor function; and (c) directly or indirectly measuring a change in NMDA function in the treated cells as compared with the untreated cells, a change being indicative that the test compound affects NMDA receptor function in a subject. In a preferred embodiment, the cells are transgenic NR2B-expressing cells. Use of such cells is advantageous because they are expected to exhibit more robust responses to the various test compounds.

The *in vivo* assay of this type is practice similarly, except utilizing animals instead of cultured cells. In a preferred embodiment, the animals are the above-described genetically altered animals with enhanced learning and memory. Use of such animals is advantageous because they are expected to exhibit more robust responses to the various test compounds.

According to yet another aspect of the invention, a method of identifying genes and gene products that affect NMDA receptor-mediated learning and memory in a subject is provided. The method comprises: (a) providing a pair of equivalent animals or cell cultures, one being transgenic and expressing an exogenous nucleic acid molecule encoding NR2B, and the other being non-transgenic for expression of an exogenous nucleic acid molecule encoding NR2B; (b) comparing profiles of gene expression (via mRNA or protein) or protein modification (covalent or non-covalent) in the transgenic and non-transgenic animals or cells; (c) isolating one or more genes or gene products whose expression or modification is altered in the transgenic animal or cells; and (d) identifying the one or more genes or gene products.

According to still another aspect of the invention, another method of identifying genes and gene products that affect NMDA receptor-mediated learning and memory in a subject is provided. This method comprises: (a) providing cells containing NMDA receptors; (b) stimulating the NMDA receptors (directly or indirectly) in a sample of the cells; (c) comparing profiles of gene expression (via mRNA or protein) or protein modification (covalent or non-covalent) in the cell sample having stimulated NMDA receptors with an equivalent cell sample wherein the NMDA receptors are unstimulated; (d) isolating one or more genes or gene products whose expression or modification is altered in the cells having stimulated NMDA receptors; and (d) identifying the one or more genes or gene products.

Other features and advantages of the present invention will be understood by reference to the drawings, detailed description and examples that follow.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Construction and biochemical characterization of transgenic NR2B mice. Fig. 1a, The construct pJT-NR2B for production of NR2B transgenic mice. Fig. 1b, Expression of NR2B transgene mRNA in transgenic mice. Lane 1: the cortex/striatum/amygdala; Lane 2, the hippocmpus; lane 3, the brain stem and the thalamus; lane 4: the cerebellum. Fig. 1c, Synaptic NMDA receptor protein level in the hippocampus (HP) and the cortex (CTX) in both transgenic lines (Tg-1 & Tg-2) and wild-type (wt). The same membrane was used for blotting with antibodies against NR1 (120 KD), NR2A (170 KD) and NR2B (180 KD), respectively. Fig. 1d, Forebrain-specific expression of NR2B transgene revealed by *in situ* hybridization. CTX, cortex; STM: striatum; HP, hippocampus; AMG: amygdala. Fig. 1e, Normal brain morphology in transgenic mice (Nissl staining). Fig. 1f, A higher magnification of the Nissl-stained transgenic hippocampus. DG, dentate gyrus; CA1, and CA3 are marked. Fig. 1g, Golgi staining of the dendritic spines of CA1 cells from wild-type (left) and transgenic mice (right). Scale bar represents 5 µm.

Figure 2. Developmental changes in NMDA current at single synapse. Fig. 2a, Confocal image depicting a dendrite with single synapses marked by FM 1-43 (arrows). The iontophoretic electrode on the right (out of the focal plan) was brought at close proximity to the FM spot to deliver glutamate (1msec). Fig. 2b, Representative example of a current-voltage relationship of glutamate-evoked response from a single synapse from a wild type neuron. At -80 to -40 mV, non-NMDA current was exclusively observed, while at more positive potentials, both non-NMDA (peak current typically observed at 3 ms post application) and NMDA current (peak current measured 30-40 ms post-application) were recorded. The proportion

of NMDA current evoked display a tyical "J" shaped I/V relation (black circles) while non-NMDA current varies linearly with the membrane potential (open circles). Fig. 2c, Representative examples of NMDA currents at +40 mV recorded from wt (light trace) and Tg mice (dark trace) at day 10, 14, and 18 in vitro culture (DIV) respectively. Insets display the same traces, normalized and expressed as a semi-log plot to emphasize decay portion of NMDA currents. A single exponential, which provided excellent fits, was used to assess the decay time τ and values for the representative traces are indicated. Fig. 2d - Fig. 2f, Averaged values for peak amplitude, decay time, and charge transfer of NMDA currents respectively. Each point represents mean ± SEM of 8 to 18 experiments per data points obtained from 18 synapses on 13 neurons from 6 wild-type mice and 31 synapses on 19 neurons derived from 9 transgenic (Tg-1 & Tg-2 mice). (*) indicates significance between wild-type and Tg mice (P<0.01, 2-tailed unpaired student's t test).

Figure 3. Selective enhancement of 10-100Hz-induced potentiation in transgenic mice. Tg-1, filled squares; Tg-2, filled triangles; wild-type (wt), open squares. Fig. 3a, Wild-type and transgenic mouse slices showed no significant difference in paired-pulse facilitation of the EPSP at various interpulse intervals. Fig. 3b, Transgenic slices had greater NMDA receptor-mediated EPSP than wild-type slices. At 1.1 mV fiber volley, the area under the $EPSP_{NMDA}$ were 124.8 ±20.6 (mV x msec) in Tg mice and 31.1 ±5.3 (mV x msec) in controls (p<0.001). Fig. 3c, A tetanic stimulation (100 Hz, 1 s) induced significantly larger potentiation in transgenic slices (Tg-1, 9 slices/6 mice; Tg-2, 6 slices/3 mice) than wild-type slices (n=10 slices/8 mice); Inset: representative records of the EPSP before and 45 min after tetanus in a wt (left) and Tg (right) slice. Fig.

3d, 10 Hz stimulation for 1.5 min produced significant synaptic potentiation in transgenic slices (Tg-1, 5 slices/5 mice; Tg-2, 4 slices/3 mice) but not wild-type slices (9 slices/9 mice). Fig. 3e, LTD induced by low-frequency stimulation (1 Hz for 15 min) is similar between wild-type and transgenic slices. Fig. 3f, Summary data for synaptic plasticity at different frequencies. For comparisons, results from our previous study of CA1-specific NMDA-R1 knockout mice (open circles, dot line) was also included.

Figure 4. Enhanced novel object recognition memory in transgenic mice. Fig. 4a, Exploratory preference in the training session. The dotted line represents the performance at the chance level (50%). The amount of time in exploring the two objects was the same between transgenic and wild-type mice. Fig. 4b, Enhanced exploratory preference in transgenic mice in retention test. Figure indicates a temporary feature of the enhanced long-term memory in the transgenic mice (Tg-1=19, Tg-2=8, wt=14). Data were expressed as mean ± s.e.m. *, p,0.05; **, p<0.01, post-hoc analysis between transgenic and wild-type mice.

Figure 5. Enhancement of both contextual- and cued-fear memory in transgenic mice. Figs. 5a-5c, Contextual conditioning 1 hr, 1 day, and 10 days after training, respectively. Figs. 5d-5f, Cued-fear conditioning 1 hr, 1 day, 10 days after training, respectively. Each point represents data collected from 8-10 mice per group (wt, Tg-1, or Tg-2). The value in each column represents percentage of freezing rate and expressed as mean ± s.e.m. *, p<0.05, post hoc analysis between wild-type and transgenic mice.

Figure 6. Transgenic mice exhibit faster fear extinction. Fig. 6a, Faster fear extinction to contextual environment in transgenic mice. Either wild-

type (n = 8) or transgenic (Tg-1, n=7; Tg-2, n=8, data were plotted together) mice were given the same single CS/US pairing training as described in figure 5 and then subjected to 5 extinction trials 24 hr after training. Fig. 6b, Faster fear extinction to the tone in transgenic mice. The value in each column represents percentage of freezing rate and data were expressed as mean ± s.e.m. *, P<0.05; **, p<0.01; ***, p<0.001, *post-hoc* analysis.

Figure 7. Enhanced performance in the hidden-platform water maze task by transgenic mice. Fig. 7a, Escape latency (mean ± s.e.m) in the water maze training (Tg-1, n=13) or wild-type mice (n=15). Fig. 7b, Place preference in the first transfer test conducted at the end of 3rd training session. Transgenic mice spent more time in the target quadrant than other quadrants, whereas control mice did not show any preference for the target quadrant at this stage. Fig. 7c, Place preference in the second transfer test carried out at the end of the 6th training session. Both transgenic and wild-type mice exhibited strong preference for the target quadrant where the hidden-platform was previously located. *, p<0.05, *post-hoc* analysis in figure 7a, and Student' *t*-test in figure 7b, between transgenic and controls.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions:

Various terms relating to the present invention are used throughout the specification and claims.

A "coding sequence" or "coding region" refers to a nucleic acid molecule having sequence information necessary to produce a gene product, when the sequence is expressed.

The term "operably linked" or "operably inserted" means that the regulatory sequences necessary for expression of the coding sequence are placed in a

nucleic acid molecule in the appropriate positions relative to the coding sequence so as to enable expression of the coding sequence. This same definition is sometimes applied to the arrangement other transcription control elements (e.g. enhancers) in an expression vector.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

The terms "promoter", "promoter region" or "promoter sequence" refer generally to transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the coding region, or within the coding region, or within introns. Typically, a promoter is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The typical 5' promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence is a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A "vector" is a replicon, such as plasmid, phage, cosmid, or virus to which another nucleic acid segment may be operably inserted so as to bring about the replication or expression of the segment.

The term "nucleic acid construct" or "DNA construct" is sometimes used to refer to a coding sequence or sequences operably linked to appropriate

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regulatory sequences and inserted into a vector for transforming a cell. This term may be used interchangeably with the term "transforming DNA" or "transgene". Such a nucleic acid construct may contain a coding sequence for a gene product of interest, along with a selectable marker gene and/or a reporter gene.

The term "selectable marker gene" refers to a gene encoding a product that, when expressed, confers a selectable phenotype such as antibiotic resistance on a transformed cell.

The term "reporter gene" refers to a gene that encodes a product which is easily detectable by standard methods, either directly or indirectly.

A "heterologous" region of a nucleic acid construct is an identifiable segment (or segments) of the nucleic acid molecule within a larger molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, coding sequence is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

A cell has been "transformed" or "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA (transgene) may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a

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plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations. If germline cells are stably transformed, the transformation may be passed from one generation of animals arising from the germline cells, to the next generation. In this instance, the transgene is referred to as being inheritable.

Other definitions are found in the description set forth below.

II. **Description**:

It has been widely held that mental and cognitive attributes, such as intelligence and memory, are the products of a variety of genetic and environmental factors. However, the present inventor has made the surprising and unexpected discovery that alteration of a single biological event, i.e., NMDA receptor-dependent modification of synaptic efficacy, significantly improves associative learning and memory in mammals. The inventor demonstrated this unifying mechanism for associative learning and memory through a genetic manipulation of the NMDA receptor in mice. As described in detail in the examples, the inventor has shown that overexpression of NMDA receptor 2B (NMDAR2B, or NR2B) in the forebrains of transgenic mice leads to enhanced activation of NMDA receptors, facilitating

synaptic potentiation in response to a 10-100 Hz stimulation. These mice exhibit enhanced capability of both learning and memory in six behavioral tasks, demonstrating a pivotal role of NR2B in gating the age-dependent threshold for plasticity and memory formation and, more broadly, demonstrating that enhanced activation of NMDA receptor function leads to marked improvement in mammalian learning and memory, through enhancement of synaptic plasticity. Thus, the NMDA receptor has now been shown to function as a "master molecular switch" for various forms of learning and memory.

The studies with NR2B transgenic animals have revealed fundamental biochemical changes in the NMDA receptor that lead to the observed improvements in learning and memory. These include the maintenance of a large single synapse peak amplitude and a prolonged channel decay, which together result in larger charge transfer through the synaptic NMDA receptor channel, and increased information transfer as exemplified by ion influx and efflux in the NMDA receptor-containing neurons. Therefore, overexpression of the NR2B transgene has resulted in the prolonged opening of the NMDA receptors for detecting coincidence and the enhanced NMDA activation in individual synapses, thus retaining several juvenile features of NMDA receptor properties.

The term "synaptic plasticity" as used herein refers to the ability of a neuron to change its communication efficacy based on past firing activity. One example is the phenomenon of long-term potentiation (LTP): upon a high frequency stimulation, the synaptic communication efficacy is potentiated, and that potentiation can last for several weeks. The term "NMDA receptor activity" refers to parameters of the NMDA receptor itself, such as channel decay time or peak amplitude of the electrochemical signal through the

channel. The inventor has show that improvements in NMDA receptor activity lead to enhanced synaptic plasticity. These two features are referred to collectively herein as "NMDA receptor function". It is the improvement or enhancement of NMDA receptor function that results in enhanced learning and memory in accordance with the present invention.

The studies with NR2B transgenic animals reported herein have shown that learning and memory is enhanced with only a modest amount of improvement of NMDA receptor function. For example, focusing on NMDA receptor activity, a 15-20% increase in length of channel decay still resulted in improved learning and memory behaviors in the transgenic animals, as compared to non-transgenic animals. Greater increases in NMDA receptor function are preferred for practice of the invention (e.g., 30%, 40%, 50% or more increase in channel decay length, peak amplitude, or gating), but the threshold appears to be relatively low.

The discoveries made in accordance with the present invention can be put to practical use in a variety of ways. One overall aspect of the invention provides a method for improving learning or memory in a mammal, which comprises altering an effective amount of the NMDA receptors in learning or memory-associated brain areas (e.g., hippocampus) in a manner that enhances synaptic plasticity in neurons containing the altered receptors.

It is believed that any means by which NMDA function can be enhanced will yield the same improvements in learning and memory as observed in the NR2B transgenic mice. Thus, altering the ratio of NR2B to NR2A in NMDA receptors via overexpression of NR2B is but one means by which such a result may be achieved. Other methods to enhance NMDA receptor function include, but are not

limited to: (1) use of small molecules that act directly or indirectly to enhance NMDA receptor function; (2) modulating expression of NMDA receptor subunits at the transcriptional (e.g., promoter), translational (which may include upregulation of upstream transcription factors) and/or post-translational level (e.g., covalent or non-covalent protein modification); (3) use of agents that act inside the cell at the intracellular domains of the NMDA receptor or one of its downstream signaling molecules, thereby modulating interaction between the receptor and its downstream targets; (4) use of agents inside the cell to stimulate the downstream gene expression as seen in the NMDA-activated transgenic mice or cells described herein; and (5) enhancing the NMDA receptor-mediated processes indirectly by modulating other neuronal receptors (e.g., the AMPA receptor, GABA receptor, serotonin receptor) or presynaptic neurotransmitter releases, thereby affecting NMDA receptor responses.

In a preferred embodiment, NR2B subunits are targeted for modulation. As exemplified herein, NR2B may be overexpressed in a desired region of the brain through genetic manipulation. In one embodiment, a subject may be stably transformed with a vector encoding NR2B subunits. Stable transformation is preferably applied to juvenile or embryonic subjects, such that the subjects enjoy enhanced synaptic plasticity through their youth and into adulthood. In a particularly preferred embodiment, germline cells of embryonic subjects are transformed, resulting in subjects that can pass the transgene along to their offspring. These types of transformation are accomplished using techniques well know to persons skilled in the art, as described in greater detail below.

Alternatively, somatic cells of subjects may be

stably or transiently transformed with a vector encoding NR2B subunits. Somatic transformation is suitable for juvenile or adult subjects, as a means to correct or reverse an existing defect or pathological condition of the brain. Such "DNA therapy", for instance would comprise targeted administration of an NR2B expression vector which, upon delivery to the target cells, would produce excess NR2B that would be incorporated into NMDA receptors. DNA therapy to transiently produce NR2B in targeted brain locations is accomplished according to methods well known in the art, as described in greater detail below.

It should be understood that the term "subject" or "patient", as used herein, refers to humans (where appropriate) and also to non-human animals. The methods of the invention may be applied to any organism having a central nervous system that contains NMDA receptors. Mammals are preferred, and humans are particularly preferred, especially wherein the methods are used to treat or alleviate a neurological pathological condition or disease.

In preferred embodiments, NMDA receptors of the forebrain are targeted for enhancement, including the cortex, striatum, hippocampal structures, hippocampal formation, amygdala, and limbic system. Other learning-associated brain areas also may be targeted, including the cerebellum and thalamic region, nucleus accumbens and basal ganglion. NMDA receptor enhancement can be used in any area of the nervous system in which the receptors are located, to achieve a variety of desirable results. Such therapy is expected to be useful for the treatment of various learning and memory disorders, including schizophrenia, Alzheimer's disease and other age-related learning or memory impairment and amnesia of all types, including memory deficit resulting from drug or alcohol

abuse, as well as for the restoration of capacity to individuals who have suffered brain damaged due to, e.g., head trauma, stroke or ischemia (e.g., by drowning).

It should also be appreciated that, since enhancement of NMDA receptor function improves learning and memory, then impairment of NMDA receptor function should impair learning and memory. Such impairment also may have therapeutic utility, and the present invention is also drawn to compositions and methods for impairing NMDA receptor function. This therapy is expected to be useful in instances where cognitive suppression is desired, such as in the management of chronic pain (a form of plasticity or memory) or for purposes of creating selective amnesia with respect to a traumatic event.

The present invention also provides genetically modified non-human animals, and cells of those animals, which have been altered in NMDA receptor function so as to display enhanced synaptic plasticity as compared with an equivalent, but unaltered animal. The genetic modification made to such animals can be one of many types, including introduction of an exogenous gene (transgenic animals as described in greater detail below), or up-regulation or down-regulation of an endogenous gene, by natural or artificial mutagenesis or by transgenic manipulation.

In a preferred embodiment, NR2B transgenic animals are provided, which are expected to be useful for a variety of purposes, as discussed in greater detail below. As exemplified by the NR2B transgenic mice described herein, these animals exhibit enhanced capabilities relating to acquiring new information (i.e., learning) and storing existing information (i.e., memory).

The term "animal" is used herein to include all vertebrate animals, except humans. It also includes an

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individual animal in all stages of development, including embryonic and fetal stages. Examples of animals preferred for use in the present invention include, but are not limited to, rodents, most preferably mice and rats, as well as cats, dogs, dolphins and primates.

A "transgenic animal" is any animal containing one or more cells bearing genetic information altered or received, directly or indirectly, by deliberate genetic manipulation at the subcellular level, such as by targeted recombination or microinjection or infection with recombinant virus. The term "transgenic animal" is not meant to encompass classical cross-breeding or *in vitro* fertilization, but rather is meant to encompass animals in which one or more cells are altered by or receive a recombinant DNA molecule, i.e., a "transgene". The term "transgene", as used herein, refers to any exogenous gene sequence which is introduced into both the somatic and germ cells or only some of the somatic cells of a mammal. This molecule may be specifically targeted to defined genetic locus, or be randomly integrated within a chromosome, or it may be extrachromosomally replicating DNA. The term "germline transgenic animal" refers to a transgenic animal in which the transgene was introduced into a germline cell, thereby conferring the ability to transfer the transgene to offspring. If such offspring in fact possess the transgene then they, too, are transgenic animals.

The transgene of the present invention includes without limitation, the entire coding region of an NR2B gene, or its complementary DNA (cDNA), or chimeric genes containing part or all of a NR2B coding region, whose expression in the forebrain is driven by a tissue specific promoter. It is preferable, but not essential, that the NR2B coding sequence used in the transgene be of the same species origin as the transgenic animal to be created.

Nucleic acid sequences encoding NR2B have been reported for several species. Examples include mouse (GenBank Accession No. U60210) and human (GenBank Accession No. NM 000834).

The promoter is comprised of cis-acting DNA sequences capable of directing the transcription of a gene in the appropriate tissue environment and, in some cases, in response to physiological regulators. The promoter preferred for use in the present invention is derived from the *αCaMKII* gene, whose activity has been demonstrated to be restricted to the forebrain region (Mayford et al., Cell **81**, 891-904, 1995). Other promoters are also known to direct the expression of exogenous genes to specific cell-types in the brain. Promoters useful for stem cell transformation, wherein tissue specificity is needed, include any promoter whose endogenous genes are expressed in the target cell of interest; e.g., the pkcγ promoter, the telencephalin promoter, the neuronal enolase promoter and the prp promoter. For somatic transformation, tissue specific promoters may or may not be needed. Thus, constitutive promoters, such as the CMV promoter or the β-actin promoter should prove useful for somatic transformation.

Methods to obtain transgenic, non-human mammals are known in the art. For general discussions, see, e.g., Joyner, "Gene Targeting," IRL Press, Oxford, 1993; Hogan et al. (Eds.), "Manipulating the Mouse Embryo - A Laboratory Manual," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1994; and Wasserman & DePamphilis, "A Guide to Techniques in Mouse Development," Academic Press, San Diego CA, 1993. One method for introducing exogenous DNA into the germline is by microinjection of the gene construct into the pronucleus of an early stage embryo (e.g., before the four-cell stage) (Wagner et al., *Proc. Natl. Acad. Sci.*

USA **78**, 5016, 1981; Brinster et al., *Proc. Natl. Acad. Sci.* USA **82**, 4438, 1985). The detailed procedure to produce NR2B transgenic mice by this method has been described (Tsien et al., *Cell* **87**, 1317-26, 1996).

Another method for producing germline transgenic mammals utilizes embryonic stem cells. The DNA construct may be introduced into embryonic stem cells by homologous recombination (Thomas et al., *Cell* **51**, 503, 1987; Capecchi, *Science* **244**, 1288, 1989; Joyner, et al., *Nature* **338**, 153, 1989) in a transcriptionally active region of the genome. A suitable construct may also be introduced into the embryonic stem cells by DNA-mediated transfection, such as electroporation (Ausubel, et al., Current Protocols in Molecular Biology, John Wiley & Sons, 1999). Detailed procedures for culturing embryonic stem cells and methods of making transgenic mammals from embryonic stem cells may be found in Teratocarcinomas and Embryonic Stem Cells, A practical Approach, ed. E. J. Robertson (IRL Press, 1987).

Other methods for producing germline transgenic animals are being developed currently. For instance, instead of eggs being the recipients of exogenous DNA, sperm are now being genetically manipulated or targeted by mutagenic agents.

In any of the foregoing methods of germline transformation, the construct may be introduced as a linear construct, as a circular plasmid, or as a viral vector which may be incorporated and inherited as a transgene integrated into the host genome. The transgene may also be constructed so as to permit it to be inherited as an extrachromosomal plasmid. The term "plasmid" generally refers to a DNA molecule that can replicate autonomously in a host cell.

Transgenic animals also may be obtained by

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infection of neurons either *in vivo, ex vivo,* or *in vitro* with a recombinant viral vector carrying an NR2B gene. Suitable viral vectors include retroviral vectors, adenoviral vectors and Herpes simplex viral vectors, to name a few. The selection and use of such vectors is well known in the art.

The present invention also provides a variety of assays and other methods, which utilize the inventors' discovery of the profound effect of NMDA receptor function enhancement on synaptic plasticity, learning and memory.

One useful assay is an *in vitro* assay for identifying compounds that enhance learning and memory by increasing expression of NR2B genes. This assay involves the following basic steps: (1) provide a chimeric DNA construct comprising an NR2B promoter operably linked to a reporter gene; (2) contact the chimeric DNA construct with a test compound suspected of up-regulating the NR2B promoter, and (3) measure expression of the reporter gene. An increase in the expression of the reporter gene indicates that the test compound will enhance learning and memory by increasing expression of NR2B genes.

The NR2B transgenic animals of the invention may be used for several *in vivo* assays. For instance, they may be used as follows for identifying compounds that enhance learning and memory by affecting expression of NR2B or activity of NMDA receptors: (1) provide a pair of equivalent animals, one being NR2B transgenic, and the other being non-transgenic; (2) treat the non-transgenic animal with a test compound suspected to affect the expression or activity of the NR2B; (3) compare (biochemically or using behavioral tests) learning and memory of the treated, non-transgenic mammal with learning and memory of the transgenic mammal (and, optionally, learning and memory of an untreated, non-

transgenic mammal). Any change in learning and memory in the treated, non-transgenic mammal that comprises the same features of learning and memory exhibited in the transgenic mammal would be an indicator that the test compound enhances learning and memory in the mammal by affecting expression of NR2B or activity of NMDA receptors.

Another *in vivo* assay, useful for identifying compounds that affect activation of NMDA receptors in a mammal, comprises the following steps: (1) provide a pair of animals; (2) treat one with a test compound suspected of affecting NMDA receptor function; and (3) directly or indirectly measure a change in activity of the treated animal as compared with the untreated animal, a change being indicative that the test compound NMDA receptor function in the animal. This assay can be modified by the use of a pair of NR2B transgenic animals, or by comparing an NR2B transgenic animal with a non-transgenic animal and/or with a transgenic "knockout" animal, whose NMDA receptors are dysfunctional or non-functional (e.g., as described by Tsien et al., 1996, *supra*).

The NR2B transgenic animals of the invention may also be used in method of identifying genes and gene products that affect NMDA receptor-mediated learning and memory. Such an assay has the following steps (1) provide a pair of equivalent animals, one being NR2B transgenic, and the other being non-transgenic; (2) compare profiles of gene expression (via mRNA or protein production) or post-translational modification) in the transgenic and non-transgenic animals; (3) isolate one or more genes or gene products whose expression is altered or which is modified in the transgenic animal; and (4) identify the one or more genes or gene products. This assay also can be modified by comparing the NMDA transgenic and non-transgenic animals with a transgenic

"knockout" animal. Another adaptation of this type of assay is to subject non-transgenic animals to chemical or electrical neuronal stimulation to enhance NMDA function, then to monitor the gene expression profile of the treated animals and compare it to the expression profile observed in the NR2B transgenic animals.

It will be appreciated that assays similar to the *in vivo* assays discussed above can be developed easily in cultured cells. For instance, cultured neuronal or non-neuronal cells may be transformed with a DNA construct for expression of NR2B, optionally together with NR1 subunit or other NR2 subunits (NR2A, NR2C, NR2D), and those cells used for various biochemical and physiological assays to assess the changes resulting from the presence of the transgene. In another embodiment, cells or tissue slices from NR2B transgenic animals may be utilized for a similar purpose.

In a simple, but preferred embodiment, non-transgenic cultured cells of a selected type are used in an assay to screen for compounds that can enhance learning and memory by improving NMDA receptor function. The cells are exposed to a test compound and NMDA receptor activity is measured by standard biochemical or electrophysiological means. If NMDA receptor function is improved in comparison to untreated cells, the test compound is suitable for further analysis as a pharmaceutical compound for enhancing learning and memory.

The discoveries made in accordance with the present invention also suggest other methods for identifying genes and gene products that affect NMDA receptor-mediated learning and memory in a subject. For instance, identifying proteins (and genes encoding them) that physically interact with the receptor or with genes encoding the receptor can yield useful information

relating to NMDA receptor signaling pathways in the cell. Such interactions may be identified by several methods known in the art, including (1) the yeast two-hybrid system, (2) phage display and (3) immunoprecipitation.

The inventor's discovery that the NMDA receptor serves as a "master switch" in learning and memory also points to diagnostic assays based on polymorphisms or mutations in the genes encoding the receptor. Such polymorphisms or mutations, which could occur in the coding region or the non-coding region, are identified in subjects outside the norm (above or below) with respect to a selected form of learning or memory, and these polymorphisms are correlated with either the enhancement or the impairment of the selected form of learning or memory. Once identified, these polymorphisms can be used as a genetic screen to predict Intelligence Quotient or other measurements of learning and memory capability, or predisposition to learning or memory disorders.

The foregoing assays and methods are only a few of many that can be developed as a result of the discoveries made in accordance with the present invention. Persons of skill in the art will find numerous additional ways to use this information, and the biological tools and animal model systems described herein.

The following examples are set forth to illustrate embodiments of the invention. They are not intended to limit the scope of the invention in any way. The examples describe experiments performed to test the notion that the NR2B subunit is crucial for implementing Hebb's rule and gating synaptic plasticity and memory. To accomplish this, the NR2B subunit was overexpressed postnatally in mouse forebrains. Of seven lines produced, the examples describe results from two

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independent lines (Tg-1 and Tg-2), which have been systematically analyzed and have been found to have similar expression patterns, levels, and nearly identical electrophysiological and behavioral phenotypes.

Example 1
Production and Basic Characterization of NR2B Transgenic Mice

The linearized NR2B transgene expression vector pJT-NR2B containing the CaMKII promoter and the NR2B transgene was obtained by digestion of pJT-NR2B with Sal and purified away from plasmid sequence. The transgenic founders were produced by pronuclear injection of the linearized DNA into C57B/6 inbred zygotes as described (Tsien et al., *Cell* **87**, 1317-26 1996). The inbred founders were crossed into either C57B/6 or CBF1 to produce F1 generation. The F2 offsprings derived from intercross between C57B/6 and CBF1 were used for various analyses. We found that F2 wild-type mice on this hybrid background consistently showed excellent learning behaviors, which are critical to any comparative behavioral studies. This mating strategy, therefore, sets a much higher standard for our behavioral enhancement experiments.

The genotypes of all offspring were analyzed by preparing tail DNAs. The 5' and 3' primers for detecting NR2B transgene SV40 polyA sequence (505 bp) were 5'-AGAGGATCTTTGTGAAGGAAC-3' (SEQ ID NO:1) and 5'-AAGTAAAACCTCTACAAATG-3' (SEQ ID NO:2), respectively. Mouse tail DNAs (about 1μg) were amplified 30 cycles (1 min, 94°C; 45 sec, 55 °C; 1 min, 72 °C) on a thermal cycler. For detecting transgene mRNA, a SV40 poly(A) tail fragment was used for Northern blot. For Western blot, the antibodies against NR1, NR2A, and NR2B were purchased from Upstate Biotechnology. Synaptic membrane

proteins were prepared from the mouse forebrain. The sample were resolved on 7.5% SDS-polyacrylamide gel followed by immunoblotting with the above antibodies respectively, detected by peroxidase-labeled secondary antibodies and the ECL detection system (NEN Life Science products). For *in situ* hybridization, mouse brains were dissected and rapidly frozen. Crytostat sections (20 µm) were prepared and postfixed for 5 min in 4% PFA in PBS buffer (pH7.5). The slices were hybridized to the [$\alpha^{35}S$] oligonucleotide probe (5'-GCAGGATCCGCTTG GGCTGCAGTTGGACCT-3'; SEQ ID NO:3), which hybridizes to sequences present in the 5' untranslated artificial intron region unique to the transgene. The detailed procedures were the same as previously described (Mayfield et al., *Cell* **81**, 891-904 1995).

RESULTS

The transgenic animals, named *Doogie*, appeared to be normal in their growth, body weights, and to mate normally. Their open field behaviors were also indistinguishable from those of wild-type littermates. In addition, we did not observe any signs of seizure or convulsion in transgenic animals. Northern blot analysis showed that the NR2B transgene expression was enriched in the cortex and the hippocampus, with little expression in the thalamus, the brainstem and the cerebellum (Fig. 1b). Western blot analysis revealed approximately a 1-fold increase in the level of cortical and hippocampal NR2B protein in transgenic mice (Fig. 1c). We have also found that there is a small increase in NR1 protein level but no change in NR2A level in these regions (Fig. 1c). This indicates that both the ratio of NR2B over NR2A in the receptor complex and the total number of the NMDA receptors may be increased.

We investigated the transgene's anatomical

distribution using *in situ* hybridization and found that the transgene was highly enriched in the cortex, striatum, hippocampus, and amygdala (Fig. 1d). At light microscopic level, we found no gross strucutural abnormalities in these transgenic animals (Fig. 1e, f). In addition, the shapes and architecture of dendritic spines of the hippocampus and the cortex are also normal (Fig. 1g).

Example 2
Electrophysiological Analyses of NR2B Transgenic Mice

METHODS:

Hippocampal cell culture and recording. Primary cultures of hippocampal neurons were prepared from individual neonatal mice (P1). Whole cell patch recordings were carried out as described elsewhere (Liu et al., *Neuron* **22**, 395-409, 1999). The composition of the FM 1-43 (Molecular probe, Eugene, Or.) staining solution was KCl 90 mM, NaCl 39 mM, Glucose 30 mM, HEPES 25 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, and FM 1-43 0.01 (adjusted to pH 7.4 with NaOH). Recordings were made with 200B integrating patch clamp amplifier (Axon Instruments) with a 1 kHz (8 pole Bessel) low-pass filter. Data were digitized at 10 kHz using a Digidata 1200B A/D converter (Axon Instruments). Glutamate currents were evoked by iontophoresis as described (Liu et al., 1999, *supra*). Briefly, following a one minute incubation in the FM1-43 solution, neurons, continuously perfused with tyrode, were visualized under confocal microscope (Olympus Fluoview) using a 40x planachromat water immersion objective. Following placement of the iontophoresis electrode, brief (1ms) glutamate pulses of varied amplitudes were delivered to an isolated FM-labeled presynaptic bouton.

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Hippocampal slice recording. Transverse slices of the hippocampus from transgenic and wild-type littermates (4-6 month old) were rapidly prepared and maintained in an interface chamber at 28°C, where they were subfused with ACSF consisting of 124 mM NaCl, 4.4 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.0 mM Na_2HPO_4, and 10 mM glucose, bubbled with 95% O_2 and 5% CO_2. Slices were kept in the recording chamber for at least two hours before the experiments. A bipolar tungsten stimulating electrode was placed in the stratum radiatum in the CA1 region and extracellular field potentials were recorded using a glass microelectrode (3-12 MΩ, filled with ACSF) also in the stratum radiatum. Stimulus intensity was adjusted to produce a response of approximately 1 mV amplitude, with an initial slope of approximately -0.5 mV/msec. Test responses were elicited at 0.02 Hz. Homosynaptic LTD was induced by prolonged low frequency stimulation (1 Hz for 15 min). LTP was induced by tetanic stimulation (100 Hz for 1 sec). Paired-pulse facilitation (PPF) of the response at various interpulse intervals (25-400 msec) was also measured. In depotentiation experiments, the stimulus to produce LTP was 100 Hz for 1 sec, delivered twice with an interval of 20 sec. This was followed by a low frequency stimulus of 5 Hz for 3 min to produce depotentiation. Data are presented as mean ± s.e.m.. One-way analysis of variance (with Duncan's multiple range test for post hoc comparison) and Student's *t*-test were used for statistical analysis.

RESULTS

To evaluate the elementary properties of the NMDA receptors in single synapses, we employed a novel single bouton recording technique (Liu et al., 1999, *supra*). Using FM 1-43 as a label of functional synaptic

sites in cultured hippocampal neurons, we positioned the tip of an iontophoretic electrode containing 150 mM glutamate adjacent to a relatively isolated synapse (Fig. 2a), and applied glutamate to determine the functional properties of glutamate receptors located in that particular synapse. Glutamate-evoked responses consisted of either AMPA current alone or both AMPA and NMDA currents depending upon the cell potential. The NMDA component was identified by its "J" shape of current-voltage relationship and long decay time (Fig. 2b). Thus, NMDA currents were isolated by clamping the cells to +40 mV to remove voltage-dependent Mg^{2+} block. While initial experiments were carried out in the presence of 5 μM DNQX to the bath solution to block AMPA receptors, we subsequently omitted the antagonist as NMDA current could clearly be isolated from the AMPA based on their respective time courses, and all the experiments were conducted in blind fashion.

We first determined the dose-response relation of the synaptic NMDA receptors to glutamate (an index of glutamate affinity for the NMDA receptor) and its voltage-dependent Mg^{2+} block, and found no difference between the two groups of mice. Since the recombinant NR2B subunit determines the decay phase of NMDA currents *in vitro*, we next measured NMDA channel decay time from currents evoked by a saturating dose of glutamate (100 nA of iontophoretic current, as determined from the dose-response relationship) in both transgenic and wild-type neurons. While we found no difference in decay time at day 10 or 14 in vitro (DIV), the decay time of the NMDA currents from transgenic neurons at day 18 DIV was 1.8 fold longer than those of wild-type neurons (depicted in the insets of Fig 2c and summarized in Fig. 2e, $p<0.005$). In addition, overexpression of the NR2B transgene resulted in the retention of the juvenile-like, single

synapse peak NMDA current amplitude over time in culture. This is in contrast to that of wild-type neurons which decreased significantly by 18 DIV [Fig 2d, significance between NR2B (n=18) and wild-type (n=8), p<0.01, significance between 14 (n=8) and 18 DIV (n=8) for wild-type p<0.01]. This suggests that the total number of the NR2B-containing NMDA receptors per single synapse is also higher than that of wild-type animals at this stage. These age-dependent changes in channel decay time and peak amplitude are consistent with *in vivo* observation that the NR2B transgene mRNA was detectable but low at P14, and gradually increased to a steady level approximately a week later.

The prolonged decay time and the maintenance of large single synapse peak amplitude should result in larger charge transfer through the synaptic NMDA receptor channel. As such, we calculated the total amount of charge transfer associated with the activation of the NMDA receptors at a single synapse by integrating the area between the time of glutamate application and 400 ms later. Results clearly indicate that, at 18 DIV, the total charge transfer through single synapse NMDA receptors was about 4-fold larger in transgenic mice than that of controls [2.5 ± 0.7 pC (n = 8) in wild-type versus 9.8 ± 1.7 pC (n = 18) in NR2B mice p<0.001] (Fig. 2f). Therefore, overexpression of the NR2B transgene has resulted in the prolonged opening of the NMDA receptors for detecting coincidence and the enhanced NMDA activation in individual synapses, thus retaining several juvenile features of NMDA receptor properties.

Since blockade of the NMDA receptors prevents the induction of major forms of plasticity such as LTP and LTD2, we examined whether the increased time window of coincidence detection via the NMDA receptors leads to the enhancement of synaptic plasticity in the CA1 region

of the hippocampus. Using the hippocampal slices prepared from the 4-6 months-old animals (in blind fashion), we first measured the NMDA-mediated field EPSPs in both adult wild-type and transgenic mice in the Schaffer collateral CA1 path. The NMDA receptor-mediated EPSPs were isolated in the presence of 10 µM CNQX and 0.1 mM Mg^{2+}. We found that the NMDA receptor-mediated field EPSPs in transgenic mice were significantly greater than those in wild-type mice, suggesting that the overexpression of NR2B has resulted in the enhancement of NMDA receptor-mediated field responses (Fig. 3b). In addition, we confirmed that the observed synaptic responses were NMDA receptor-dependent because it was sensitive to NMDA receptor antagonist, 100 µM AP-5 (n=3).

We then studied AMPA receptor-mediated responses and found no difference in AMPA-mediated field EPSPs between transgenic (n = 62 slices/14 mice) and wild-type littermates (n = 50 slices/16 mice, data not shown). Furthermore, paired-pulse facilitation, which gives an indication of presynaptic function, was similar between transgenic mice (n = 11 slices/7 mice) and wild-type hippocampal slices (n = 7 slices/5 mice) (Fig. 3a). These results suggest that both presynaptic function and postsynaptic AMPA receptors are normal in transgenic animals.

To provide overall assessment of bidirectional synaptic plasticity in 1-100 Hz range (Dudek et al., *J. Neurosci.* **13**, 2910-2918, 1993), we conducted a series of LTP/LTD experiements in the Schaffer collateral CA1 pathway in blind fashion. We found that a single tetanic stimulation (100 Hz, 1 s) typically evoked smaller but reliable potentiation in 4 to 6 month-old control slices in comparison to that in younger adult slices. However, the same stimulus evoked significantly larger potentiation in transgenic slices (Fig. 3c; transgenic, n

= 9 slices/6 mice; wild-type mice, n = 10 slices/8 mice). The enhancement of potentiation was not due to changes in inhibitory GABAergic mechanisms since it was similarly observed in the presence of 100 µM picrotoxin (transgenic, n = 4 slices/3 mice, mean 241.1 ± 48.2%; wild-type, n = 5 slices/5 mice, mean 140.3 ± 19.1%; P<0.05 compared to transgenic mice). Moreover, the enhanced LTP was completely blocked by the application of NMDA receptor antagonist, AP-5 (100 µM), in the bath. In addition, we noted that in contrast to the normal fast decay of fEPSP in control slices during the first 10 minutes after tetanic stimulation, there was no decay at all, and it remained maximally potentiated, a feature resembled with the juvenile LTP observed in postnatal day 15 animals (Harris et al., *J. Physiol.* (Lond) **346**, 27-48, 1984).

The enhanced long-term potentiation in the transgenic slices was further observed when we applied prolonged, repetitive stimulation (10 Hz) to Schaffer-CA1 path. We found that while the 10 Hz stimulation for 1.5 min (900 pulses) did not induce reliable synaptic potentiation in control animals (n = 9 slices/9 mice), it was fully capable of evoking robust synaptic potentiation in transgenic slices (n = 5 slices/5 mice) (Fig 3d). However, repetitive stimulation delivered at 5 Hz for 3 min with the same amount of pulses (n = 900) did not produce significant synaptic potentiation in both groups of mice (transgenic, n = 5 slices/5 mice; mean 96.8 ± 20.3%; wild-type, n = 5 slices/5mice, mean 85.0 ± 16.4%).

We then investigated long-lasting synaptic depression-induced by low frequency (Bear et al., 1994, *supra*; Dudek et al., 1993, *supra*). We found that 1 Hz stimulation produced a similar LTD in both control animals (n = 6 slices/6 mice, 76.0 ± 9.3%) and transgenic mice (n = 8 slices/7 mice, 76.8 ± 13.6%) (Fig. 3e). In addition,

we also examined synaptic depression using another protocol in which low-frequency stimulation (5 Hz, 3 min) was applied 5 min after strong tetanic stimulation (100 Hz, 2 x 1 s) (Stubli & Chun, *J. Neurosci.* **16**, 853-60, 1996). Again, similar depression or depotentiation was induced in slices of transgenic mice (n = 4 slices/4 mice; 129.3 ± 12.9%) and wild-type mice (n = 6 slices/6 mice, 111.1 ± 14.8%). As summarized in Fig. 3f, these results show that the enhanced NMDA receptor activation in transgenic mice results in selective enhancement of long-lasting synaptic potentiation evoked by the 10-100 Hz frequency stimulation.

What are the effects of the selective enhancement of 10-100 Hz LTP responses on learning and memory? Since forebrain neurons often fire in this range during behavioral experience (e.g. hippocampal neurons fire in 4-12 Hz range, known as the θ rhythm, whereas various cortical neurons oscillate in 20-60 Hz, known as the γ frequency), it is likely that selective enhancement of potentiation above 10 Hz in the transgenic mice could be particularly meaningful. We previously demonstrated that the conditional knockout of the NMDA receptor 1 subunit in the CA1 region leads to complete loss of synaptic changes in the 1-100 Hz frequency range (Tsien et al., *Cell* **87**, 1327-38, 1996) (Fig. 3f) and impairs performance in spatial water maze. This indicates that the normal frequency-response in this range is essential for learning and memory. A systematic downward shift (producing LTD) in this specific range can cause learning impairment (Mayfield et al., 1995, *supra*), whereas an upward shift (producing LTP) in all frequencies (1-100 Hz) is also deleterious to spatial learning (Migaud et al., *Nature* **396**, 433-439, 1998). Therefore, these observations collectively point to the importance of normal 1-100 Hz responses for learning and memory.

Example 3
Behavioral Analyses of
NR2B Transgenic Mice

METHODS

Behavioral tests. Adult transgenic and wild-type mice (3-6 months-old littermates) were used. Mice were maintained under the standard condition (23±1 °C, 50±5 % humidity) in the animal facility. All experiments were conducted in a soundproofed and specialized behavior room. All experimenters were blind to the genotype of the individual animal.

Novel Object Recognition Task. The apparatus, an open-field box (20 x 20 x10 high inches), was constructed from plywood and painted black with non-toxicity paint. Before training, mice were individually habituated by allowing them to explore the open-field box for 5 min per session for three sessions per day and for 3 days. During training session, two novel objects were placed into the open-field 14 inches away from each other (symmetrically) and then the individual animal was allowed to explore for 5 min. Exploring to object was considered when the head of animal was facing the object within 1 inch away from the object or any part of the body except the tail was touching the object. The time spent to explore each object was recorded. The animals were returned to their home cages immediately after training. During retention test, the animals were placed back into the same open-field box again after certain intervals, and allowed to explore freely for 5 min. Now, one of the familiar objects used during training was replaced by a novel object. All objects were balanced in term of physical complexity and were emotionally neutral. Moreover, the open-field and objects were thoroughly cleaned by 70% alcohol after each session to avoid possible instinctive odorant cues. A preference index, a

ratio of the amount of time spent exploring any one of the two items (training session) or the novel object (retention session) over the total time spent exploring both objects, was used to measure recognition memory. Two-way ANOVA (group X retention time) and *post hoc* Dunnett's test were used to determine genotype effects on the behavioral responses.

Fear Conditioning Task. The fear conditioning shock chamber and the TruScan multi-parameter activity monitors were used (Coulbourn Instrument). Briefly, it consists of a shock chamber (10 X 10 X 15 inches high) with a 24-bar inescapable shock grid floor, a multi-tone producer and speaker, an electrical-shock producer, a photobeam-scanner, and a workstation. The walls of the chamber are transparent, thus freezing responses of mice in the chamber could be observed by experimenters from the peep window on the curtain. Before training, animals were individually habituated to the chamber a day before the experiment for 5 min per session and three sessions total. Conditioned stimulus (CS) used was an 85dB sound at 2800Hz, and unconditioned stimulus (US) was a continuous scrambled foot shock at 0.75 mA. During the training, mice were put individually into the chamber and allowed to explore the environment freely for 3 min, and then were exposed to the CS for 30 sec. At the last 2 sec of the CS, the US was delivered for 2 sec. After the CS/US pairing, the mice were allowed to stay in the chamber for another 30 sec and then returned to their homecages immediately. Throughout these procedures, freezing responses were recorded simultaneously experimenters using a 5-sec interval time-sampling method as well as the photobeam-scanner system. Freezing was judged as complete immobile of the body except for the respiratory movements. Freezing response during the 30 sec after shock was recorded as immediate freezing.

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During the retention test, each mouse was placed into the shock chamber and freezing response was recorded for 3 min in this context (contextual conditioning). Subsequently, the mice were put into a novel chamber (triangular box with a smooth flat floor and yellow-black walls) and monitored for 3 min before the onset of the tone (pre-CS). Immediately after that, a tone identical to that in the training session was delivered for 3 min and freezing responses were recorded (cued conditioning). Two-way ANOVA (group X retention time) and *post hoc* Dunnett's test were used to determine genotype effects on the behavioral responses.

<u>Fear Extinction Experiment</u>. Another groups of transgenic and control mice were used for this experiment. Twenty-four hours after training as described above, the mice were given a first extinction trial. Each extinction trial consisted of contextual and cued extinction. The mice were first put individually into the shock chamber and observed for 3 min in the absence of electric shock (US) for the measurement contextual extinction. Then, the mice were transferred into a novel box for the measurement of cued fear extinction. The freezing responses were observed for 3 min in the absence of the tone (pre-CS) and subsequently with the identical tone used in the training session for another 3 min. Following this, the 4 same extinction trials were given at an interval of 2 hr and freezing responses were recorded throughout the texts. Two-way ANOVA (group X extinction trial) and *post hoc* Dunnett's test were used to determine genotype effects on the freezing responses.

<u>Water Maze Task</u>. The apparatus for water-maze is consisted of a circle pool (1.2 m in diameter). The procedure was essentially the same as described previously (Tsien et al., 1996, *supra*). The training

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protocol consisted of 6 sessions (4 trials/session/day). The movement of mice was tracked by a videocamera, and the escape latency to the platform was recorded. One-way ANOVA and *post hoc* Dunnett's test were used to determine genotype effects on the escape latency. In addition, two transfer tests were performed. The first one was carried out at the end of third session and the second one at the end of the last session. During the transfer test, the platform was removed and the mice were allowed to swim in the pool for 60 sec. The time spent in each quadrant was recorded. Student's *t*-test was used to determine genotype effect on the spatial preference.

RESULTS

To define whether the selective enhancement of 10-100 Hz responses represents an optimal plasticity curve, we thus conducted various learning tasks with clear relationships to the forebrain regions, and all the behavioral experiments were performed in a blind fashion with respect to the genotype of the individual mouse.

We first used the novel object recognition task to measure visual recognition memory, which is evolutionarily conserved in various species ranging from humans to rodents and requires the hippocampus (Reed & Squire, *Behav. Neurosci.* **111**, 667-75, 1997; Myhrer, *Behav. Neurosci.* **102**, 356-62, 1988; Mumby et al., *Behav. Neurosci.* **110**, 266-81, 1996). To increase the difficulty of this task, we used a 5-min training protocol (see methods). In training session, there was no significant difference in the amount of time spent on exploring the two objects as shown by the exploratory preference (Fig. 4a), indicating both types of mice have the same levels of motivation and curiosity for exploring these two objects. During retention tests, one of the familiar object used in the training session was replaced with a

third novel object, animals were allowed to explore for 5 min. Both transgenic and wild-type mice exhibited the similar levels of preference toward the novel object at the 1hr retention test (Fig. 4b). This suggests that all groups possessed the same capability to retain the memory of the old object for 1 hour. However, when retention tests were conducted either 1 day or 3 days later (Fig. 4b), both transgenic lines exhibited much stronger preference for the novel object than wild-type mice [$F(2,38) = 5.448$, $p < 0.01$], indicating that transgenic mice have better long-term memory. A *post hoc* analysis by using Dunnett's test reveals a significant difference between wild-type and either transgenic line at 1-day ($p < 0.01$) or 3-day retention test ($p < 0.01$), but not between the two transgenic lines. The observed enhancement of long-term memory is, thus, independent of transgene integration locus. It should be noted that, however, by 1 week after training, the preference in transgenic mice also returned to the basal level.

We then assessed two forms of associative emotional memories in these mice: contextual and cued fear conditionings. Animals learn to fear a neutral conditioned stimulus (CS; such as a tone) which was previously paired with aversive unconditional stimulus (US; such as foot shock) or a context in which the animals were conditioned by the pairing of CS and US. It has been shown that contextual fear conditioning is hippocampal dependent whereas cued fear conditionings is hippocampal independent (Phillips & LeDoux, *Behav. Neurosci.* **106**, 274-85, 1992). These two types of fear conditioning require the activation of the NMDA receptors (Kim et al., *Behav. Neurosci.* **106**, 591-6, 1992; Davis et al., In: The psychology of learning and memory (Bower GH. Ed). New York: Academic Press (1987).

Both contextual and cued conditioning were measured at 1 hr, 1day, and 10 days after training using separate batches of animals. We first analyzed contextual fear memory, as shown in Fig 5a-c, transgenic mice consistently exhibited much stronger freezing responses. One-way ANOVA indicates no significant difference in immediate freezing between wild-type and transgenic mice, a significant group difference, however, is found when tested 1 hr [$F(2,24) = 5.062$, $p < 0.05$], 1 day [$F(2,25) = 5.223$, $p < 0.05$], and 10 days [$F(1.18)=4.576$, $p<0.05$]. Further *post hoc* analysis reveals the significant difference between wild-type and either transgenic line ($p < 0.05$, repsectively), but not between these two transgenic lines.

Since the transgene is also abundantly expressed in the amygdala and the cortex, we then examined the cued fear conditioning. One-way ANOVA indicated that freezing in response to the tone was also significantly elevated in transgenic mice than that in controls when tested at 1 hr [$F(2,24) = 4.672$, $p < 0.05$], 1-day [$F(2,25) = 5.518$, $p < 0.01$], 10-days [$F(1.18)=6.498$, $p<0.05$] after training (Fig. 5d-f). A *post hoc* analysis shows the significant difference between wild-type and either transgenic line ($p<0.05$, respectively). The enhanced contextual and cued fear memories in transgenic mice were not due to an altered nociceptive responses since the minimal amount of current required to elicit three stereotypical behaviors: flinching/running, jumping, and vocalizing were similar between wild-type and transgenic mice.

We then conducted two additional experiments to measure emotional learning using the fear extinction paradigm (Falls et al., *J. Neurosci.* **12**, 854-863, 1992). It is known if the animals were repeatedly exposed to the context or the CS (tone) without the presence of US

(shock), the context or CS will lose its ability to produce the fearing responses. The reduction in conditioned fear is referred as fear extinction and is an NMDA-dependent process (Falls et al., 1992, *supra*). It is thought to involve the formation of a new memory rather than the passive decay or erasure of the original memory because the original associations remain intact following extinction. We examined the fear extinction using a 5-extinction trial paradigm. When we measured the initial fear response 24 hr after training, again we observed much stronger fear responses in transgenic mice than that of controls in either contextual or cued fear conditioning (Fig. 6). Remarkably, transgenic mice exhibited much less freezing during subsequent exposures to either the context or the tone than that of wild-type mice (Fig. 6a,b). Two-way ANOVA indicated that while both wild-type and transgenic mice decreased their freezing responses to contextual extinction [$F(4,80) = 86.247$, $p < 0.001$] or cued extinction [$F(4,80) = 78.415$, $p < 0.001$], a significant group difference existed between transgenic and wild-type mice in contextual extinction [$F(2,20) = 8.595$, $p < 0.01$] or in cued extinction was observed [$F(2,20) = 7.778$, $p < 0.01$]. A *post hoc* analysis revealed a significant difference in the freezing responses between wild-type and transgenic mice at the second or third extinction trial in either contextual conditioning ($p < 0.05$, respectively) or cued conditioning ($p < 0.05$, respectively). The similar faster fear extinction was also observed if the experiments were conducted 1 hr after fear conditioning. Therefore, transgenic mice are quicker to learn to disassociate the previous paired event.

Finally, we tested spatial learning in transgenic mice using the hidden-platform water maze, which requires the activation of NMDA receptors in the

hippocampus (Tsien et al., 1996, *supra;* Morris et al., *Nature* **24**, 681-3, 1982). As shown in Fig. 7, the latency to escape to the platform in both wild-type and transgenic mice decreased following the training sessions. However, a significant group difference was observed throughout sessions [$F(1,26) = 9.655$, $p < 0.01$], indicating that spatial learning in transgenic mice was faster than that in wild-type mice. Moreover, a *post hoc* analysis reveals a significant difference at third session ($p < 0.05$), confirming a better learning in transgenic mice. In addition, the enhanced spatial learning in transgenic mice was also evident in the first transfer test conducted after the third training session. In comparison with that of controls, transgenic mice already exhibited clear preference for the targeted quadrant in which the platform was previously located ($p < 0.05$; Student's *t* tests) (Fig. 7b). With additional training, control mice indeed showed the same level of preference in comparison with transgenic mice as measured by either escape latency or place preference in the second transfer test after the last (6^{th}) session. Therefore, these results have demonstrated that these transgenic mice were able to outperform their wild-type littermates in this spatial task.

The present invention is not limited to the embodiments described and exemplified above, but is capable of variation and modification within the scope of the appended claims.